



RESEARCH NOTE

xNgn2 induces expression of predominantly sensory neuron markers in *Xenopus* whole embryo ectoderm but induces mixed subtype expression in isolated ectoderm explants [version 1; referees: 1 approved with reservations]

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Abstract

Proneural basic-helix-loop-helix (bHLH) proteins, such as Neurogenin2 (Ngn2) and Ascl1, are critical regulators at the onset of neuronal differentiation. Endogenously they have largely complementary expression patterns, and have conserved roles in the specification of distinct neuronal subtypes. In *Xenopus* embryos, xNgn2 is the master regulator of primary neurogenesis forming sensory, inter- and motor neurons within the neural plate, while xAscl1 is the master regulator of autonomic neurogenesis, forming noradrenergic neurons in the antero-ventral region of the embryo. Here we characterise neuronal subtype identity of neurons induced by xNgn2 in the ectoderm of whole *Xenopus* embryos in comparison with xAscl1, and in ectodermal “animal cap” explants. We find that the transcriptional cascades mediating primary and autonomic neuron formation are distinct, and while xNgn2 and xAscl1 can upregulate genes associated with a non-endogenous cascade, this expression is spatially restricted within the embryo. xNgn2 is more potent than xAscl1 at inducing primary neurogenesis as assayed by neural-β-tubulin. In ectoderm of the intact embryo, these induced primary neurons have sensory characteristics with no upregulation of motor neuron markers. In contrast, xNgn2 is able to up-regulate both sensory and motor neuron markers in naïve ectoderm of animal cap explants, suggesting a non-permissive environment for motor identity in the patterned ectoderm of the whole embryo.

Keywords

Neurogenin2, neurogenesis, *Xenopus*, bHLH, proneural, sensory, motor.

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Introduction

Proneural proteins are members of the basic-helix-loop-helix (bHLH) family of transcription factors, with a conserved role in driving and coordinating the various stages of neurogenesis¹. In addition to neuronal specification, the concept of subtype determination by proneural factors was first established for *achaete* and *atonal* in *Drosophila*². Vertebrate homologues *Ascl1* and Neurogenin2 (Ngn2) also show largely complementary patterns of expression, with distinct roles in neuronal subtype and neurotransmitter phenotype throughout the nervous system^{1,3}.

In *Xenopus*, xNgn2 (also known as X-ngnr-1) is the master regulator of primary neurogenesis⁴, during which neurons develop in the trigeminal ganglia and in three bilateral stripes within the neural plate to form sensory, inter- and motor neurons for coordination of larval reflexes⁵. This is analogous to the role of mammalian Ngn2 in specification of sensory and motor neurons in the dorsal root ganglia and ventral spinal cord, respectively⁶. Induction of primary neurons in *Xenopus* embryos by proneural transcription factors, identified by pan neuronal marker neural- β -tubulin⁷, is often used as a measure of proneural activity and activation of a generic neuronal pathway. What is less well documented is whether these induced neurons additionally acquire a subtype identity, and whether that identity differs depending on the inducing proneural factor.

In this short study, we focus on the key *Xenopus* proneural protein xNgn2 and define xNgn2 subtype specification during induced neurogenesis in the ectoderm of whole embryos and in isolated ectodermal “animal cap” explants.

Methods

Animal care

All work has been carried out under UK Home Office Licence and has passed an Institutional ethical review committee assessment at the University of Cambridge.

Plasmids and constructs

Wild-type *Xenopus Ngn2* (Genbank accession number [NM001088333](#)) and wild-type *Xenopus Ascl1* (Genbank accession number [NM001085778](#)) were subcloned into pCS2+ between BamH1 and Xho1 sites using primers:

5_BamH1_xNgn2 = GATCGGATCCACCATGGTGCTGCTCAAGTGC
 3_Xho1_xNgn2 = GATCCTCGAGTCAAATGAAAGCGCTGCT
 5_BamH1_xash1 = GATCGGATCCACCATGGACAAGTGGCTGCGC
 3_Xho1_xash1 = GATCCTCGAGTCAGAACCAAGTGGTGAAGTC

Xenopus laevis embryo manipulation

All efforts are made to ameliorate suffering to any animal. For example, the colony of approximately 80 *X.laevis* females are housed and cared for by a dedicated team of animal technicians operating under Home Office Licence. Each experiment requires eggs from 2 or 3 females (depending on N = 2 or 3) and females are used on rotation within the colony

with at least a 3-month rest period after laying. A single male frog is sacrificed under humane conditions and Home Office Licence to provide testes for at least 16 experiments. Embryos obtained from fertilised eggs are used for the experiments and development is stopped 48 hours post fertilisation when embryos reach late neurula stage and prior to formation of tadpoles.

Thus, *X. laevis* eggs were obtained by standard hormone methods of induction, and subsequently fertilised *in vitro*. pCS2+ constructs were linearised and capped mRNA was transcribed *in vitro* using the SP6 mMessage mMachine® kit (Ambion). Embryos were injected with mRNA as indicated in the *Results* section, with GFP (for qPCR) or β -gal (ISH) as lineage tracers. Embryos were cultured at 18°C in Ficoll solution and staged according to [8](#). At stage 17–20, embryos were either snap-frozen for qPCR analysis or fixed in MEMFA for 90 minutes, as described in [9](#).

Whole mount *in situ* hybridisation (ISH)

Dig-oxigenin-labelled anti-sense probes were synthesised from plasmids *X.laevis* neural- β -tubulin¹⁰, *X.laevis* xHox11L2¹¹, *X.laevis* xHb9¹², *X.laevis* xHand2¹³ and *X.laevis* xPhox2a¹³. Whole mount ISH was performed as described in [9](#).

Quantitative real-time PCR (qPCR)

GFP expression was used to confirm successful injection and samples of four embryos were snap frozen. Whole embryo RNA was extracted using the RNeasy® Mini kit (Qiagen) and template cDNAs synthesised with the QuantiTect® Reverse Transcription Kit (Qiagen). qPCR was performed using the Quantifast® SYBR Green PCR kit (Qiagen) in a LightCycler® 480 (Roche). Thermal cycling conditions: 95°C for 5 minutes, then 45 cycles of 95°C for 10s, 60°C for 10s and 72°C for 20s. Primers provided in [14](#), see Data availability section for details.

Explant culture

For animal cap experiments, embryos were injected at the one cell stage and cultured in Ficoll solution at 14°C overnight. At stage 8, ectodermal explants from the animal pole of embryos (animal caps) were harvested in 1x MBS and subsequently cultured in 0.7x MBS at 14°C in 6-well plates with an agarose base, until corresponding whole embryos reached the desired stage. 18 caps per category were snap frozen for qPCR analysis.

Data analysis

For ISH images, representative images are shown from independent experiments and the N numbers reported refer to the total number of embryos per category. For qPCR data, mRNA expression was normalised to expression of reference gene (EF1 α for whole embryos and ODC1 for animal caps) and mRNA levels in the injected embryos were calculated relative to stage-matched uninjected controls. Mean values are plotted and error bars show the standard error of the mean from N independent experiments. Statistical significance was calculated by a paired two-tailed student T test in Microsoft Excel; NS = not significant; * = p < 0.05; ** = p < 0.025; *** = p < 0.0125.

Results

Over-expression of xNgn2 induces primary neurons with sensory characteristics in the ectoderm of whole embryos

xNgn2 and downstream proneural protein xNeuroD4 (also known as Xath3) are endogenous mediators of the primary neuron cascade, with some homology to *Drosophila atonal*, and are capable of inducing lateral ectoderm tissue to express neuronal marker neural- β -tubulin^{4,15}. Previous work suggests that these ectopic neurons have characteristics of cranial sensory and Rohon-Beard sensory neurons^{9,11}, consistent with a report from over-expression of Ngn1 in zebrafish¹⁶. In contrast, xAscl1 is normally transiently expressed in autonomic neuron precursors in the antero-ventral region of the embryo, and over-expression of xAscl1 leads to up-regulation of autonomic neuron markers over the ventral ectoderm^{13,17}.

In mammalian systems, Ngn2 is considered to have a permissive role in neuronal fate specification with subtype influenced by cellular context; for example, while Ascl1 mutant mice show defects in autonomic neurogenesis¹⁸, if Ngn2 is expressed from the Ascl1 locus this defect can be partly rescued⁶, and Ngn2 can promote autonomic rather than sensory identity in dissociated neural tubes in culture if BMP levels are elevated¹⁹. To further explore induction of the primary and autonomic neuron cascades by xNgn2 in comparison to xAscl1 in *Xenopus* embryos, mRNA encoding either xNgn2 or xAscl1 were over-expressed in one cell stage embryos with subsequent analysis by qPCR at stage 17 (Figure 1A). xBrn3d²⁰ and xHox11L2²¹ were used as markers of sensory neurons; xVsx1²² was used as an interneuron marker; xLim3²³ and xHb9¹² were used as motor neuron markers; xHand2¹⁷ and xPhox2a²⁴ were used as autonomic (noradrenergic) neuron markers; xPtf1a²⁵ was used as an inhibitory (GABAergic) neuron marker.

xAscl1 is not a potent inducer of primary neurogenesis, and at this level of over-expression, xAscl1 achieves only a modest two-fold increase in N- β -tubulin (Figure 1A); higher doses of xAscl1 are required to induce ectopic expression more widely across the dorsal ectoderm (Figure 1C). In contrast to relatively weak N- β -tubulin induction, there is a substantial induction of autonomic markers, with a five-fold increase in xHand2 and a 30-fold increase in xPhox2a. *In situ* hybridisation (ISH) for xHand2 confirms this upregulation is in the antero-ventral region of the embryo (Figure 1D) and thus spatially distinct from dorsally located N- β -tubulin expression, demonstrating that these are separate and independent cascades restricted to specific regions of the embryo.

Compared to xAscl1, an equivalent 45pg dose of xNgn2 mRNA induces a much greater 10-fold increase in N- β -tubulin, accompanied by a 20-fold increase in both sensory neuron markers xBrn3d and xHox11L2 (Figure 1A). In contrast, minimal changes are seen in motor neuron markers xLim3 and xHb9, thus indicating that the induced N- β -tubulin positive neurons in the ectoderm are adopting a sensory identity, even though Ngn2 is required for both sensory and motor fate during normal development of the neural tube^{4,6}. Ectopic expression of both xNgn2 and xAscl1 result in prominent inhibition of

the interneuron marker xVsx1, suggesting a re-specification of endogenous interneurons from the intermediate stripe on the neural plate; a finding noted previously for the interneuron marker xPax2¹¹. Focussing on autonomic neuron markers, while a 15-fold increase is seen in xPhox2a expression, there is only a small two-fold increase in xHand2 mRNA in response to ectopic xNgn2. Interestingly, ISH for xPhox2a expression (Figure 1E) reveals its up-regulation by xNgn2 is restricted ventrally in the embryo, thus as with xAscl1, the expression of these autonomic genes is spatially restricted from the primary neuron cascade. Furthermore, the relative potency of xNgn2 and xAscl1 in these two cascades reflects their endogenous functions; i.e. xNgn2 shows greater potency for induction of primary neurons while xAscl1 is more potent in the generation of autonomic neurons^{4,13}.

The general inability to induce expression of motor neuron genes in the lateral ectoderm has been reported previously^{9,11}, but may reflect a requirement for a higher level of xNgn2. Alternatively, motor marker up-regulation may occur at a later stage of development than previously assayed. Focusing on comparison between sensory versus motor neuron markers, the experiment was repeated assaying embryos at later stages of development (data from stage 20 embryos shown in Figure 1B) and testing a range of xNgn2 doses. With each two-fold increase in xNgn2 mRNA, there is a clear step-wise increase in N- β -tubulin expression, with a parallel increase in sensory neuron markers, but motor neuron markers do not increase even at high doses of xNgn2 mRNA. To compare the spatial pattern of distribution of ectopic neurons, stage 18 embryos were assayed by ISH for xHox11L2 and xHb9 as sensory and motor markers respectively (Figure 1F). xHb9 expression is confined to the neural tube region as in uninjected embryos, but the pattern of xHox11L2 expression mirrors the pattern of N- β -tubulin with extensive expression throughout the lateral epidermis outside the neural tube. Thus, in whole embryos, neurons induced ectopically by xNgn2 generally display sensory characteristics, while there is also some up-regulation of autonomic marker xPhox2a in the ventral epidermis, but ectopic motor neurons are not produced.

Over-expressed xNgn2 upregulates sensory and motor markers in animal cap explants

The relative lack of induction of ectopic motor neurons in the ectoderm region of whole embryos may be due to a non-permissive environment for this subtype cascade. This may occur if the endoderm contains an inhibitory signal, or lacks a necessary inductive signal; both of which may regulate epigenetic availability of promoters or necessary cofactors such as the LIM-HD proteins that are required for motor neuron identity in the mammalian ventral spine²⁶. To explore the activity of xNgn2 in a naïve environment in isolation from regional patterning or inductive signals in the developing embryo, we explored induction of neurons by xNgn2 in animal cap explants cultured *in vitro*²⁷.

Embryos were injected at the one cell stage with 45pg of xNgn2 mRNA, animal caps were excised from the roof of the

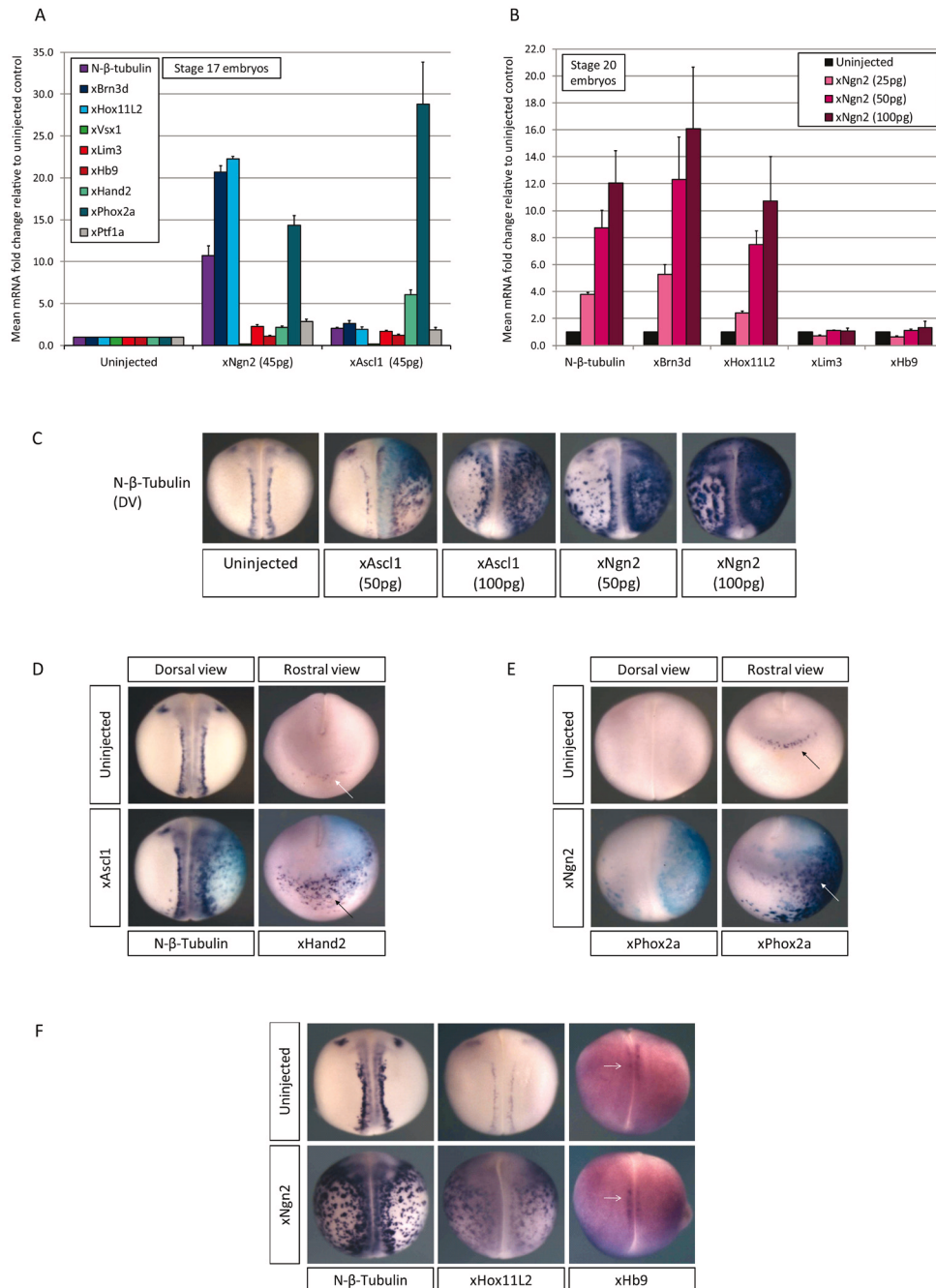


Figure 1. Over-expressed xNgn2 induces predominantly sensory neurogenesis in whole embryos. (A) One cell stage embryos were injected with 45pg mRNA encoding either xNgn2 or xAscl1. At stage 17, embryos were assayed by qPCR for expression of a range of neuronal subtype markers; [N=3]. Sensory neuron markers are induced by xNgn2 in parallel to the level of N-β-tubulin. xPhox2a is up-regulated but without induction of xHand2 that is co-expressed in autonomic neurons. (B) qPCR data from stage 20 embryos injected with increasing amounts of xNgn2 mRNA. [N=3]. A step-wise increase in N-β-tubulin is seen with parallel increase in sensory markers but no change in motor markers. (C–E) Embryos were unilaterally injected at the two-cell stage with mRNA as indicated and representative images of embryos at stage 18 are shown following *in situ* hybridisation; injected side to the right, stained with pale blue β-gal tracer. (C) N=49–61 embryos in each category in three independent experiments. DV, dorso-ventral view. xNgn2 is more potent than xAscl1 in inducing N-β-tubulin expression. (D) N=36–42 embryos in each category in two independent experiments. Endogenous xHand2 expression (white arrow) and induced xHand2 expression (black arrow) are confined to the antero-ventral region of the embryo. (E) N=31–39 embryos in each category in two independent experiments. Endogenous xPhox2a expression (black arrow) and induced xPhox2a expression (white arrow) are confined to the antero-ventral region of the embryo. (F) One cell stage embryos were injected with 45pg xNgn2 mRNA and representative images of embryos at stage 18 are shown following *in situ* hybridisation. N=21–26 embryos in each category in two independent experiments. The pattern of ectopic N-β-tubulin expression is mirrored by the pattern of ectopic xHox11L2 expression while there is no induction of motor marker xHb9 (white arrow).

blastocoel in stage 8 embryos and were then cultured until the corresponding whole embryos reached stage 17. Four independent repeat experiments were performed and in qPCR assays, N- β -tubulin and xBrn3d are found to be dramatically upregulated although the extent varies between experiments (Figure 2A), while additional markers are upregulated to a lower extent, replotted in Figure 2B using a different scale. In the naïve environment of animal caps, and in contrast to the results obtained with whole embryos, xNgn2 significantly upregulates a comparable seven to eight-fold increase in sensory marker xHox11L2 and motor marker xHb9, while trends indicating four to five-fold increases in xLim3 and interneuron marker xVsx1 are just outside statistical significance. A similar increase

is seen in xPhox2a but induction of xHand2 is not observed. Taken together, naïve caps upregulate a mix of all three primary neurons subtypes (motor, sensory and interneuron) in response to ectopic xNgn2 expression.

Animal caps expressing xNgn2 were also assayed for expression of regional neural markers using Otx2 as a fore/midbrain marker²⁸, En2 as a caudal midbrain marker²⁹, Krox20 as a hindbrain marker³⁰ and XlHbox6 as a posterior neural plate marker³¹. Small changes in expression of regional markers may indicate a trend towards more anterior CNS than posterior neural plate identity but statistical significance of any changes compared to naïve caps is not reached, so firm conclusions cannot be drawn.

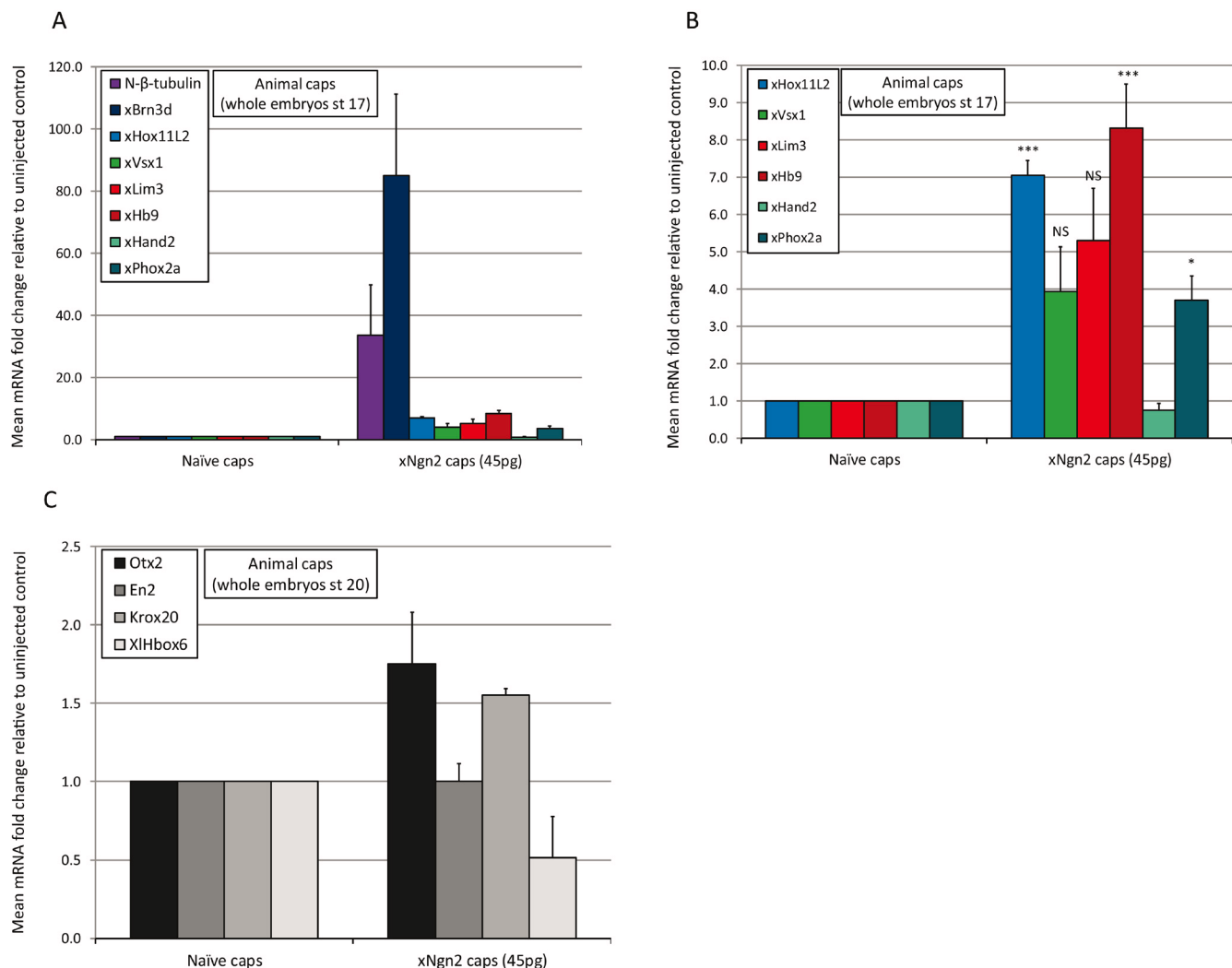


Figure 2. Over-expressed xNgn2 upregulates sensory and motor markers in animal cap explants. One cell stage embryos were injected with 45pg mRNA encoding xNgn2. At stage 8 animal caps were cut and cultured until the respective whole embryos were at stage 17 (A,B) or stage 20 (C), and assayed for gene expression by qPCR, relative to uninjected naïve caps. (A) Gene expression for the full range of subtype markers; N- β -Tubulin and xBrn3d are dramatically up-regulated but variable in absolute magnitude in the cap assays [n=4]. (B) Data replotted without N- β -Tubulin and xBrn3d. Statistical significance was calculated relative to naïve caps by a paired two-tailed student T test; NS, not significant; * = $p < 0.05$; *** = $p < 0.0125$. In contrast to whole embryos, xNgn2 upregulates motor neuron markers in animal caps to an equivalent level as sensory marker xHox11L2. (C) Expression of regional markers show a trend to more anterior rather than posterior neuronal identity [N=2].

Conclusions

In this short study we have characterised xNgn2 proneural activity in the ectoderm of whole embryos in comparison to the activity of xAscl1, as well as its activity in isolated ectodermal animal cap explants. In whole embryos, ectopic primary neurons generated show predominantly sensory characteristics with the pattern of sensory marker xHox11L2 mirroring that of N- β -tubulin. Mammalian Ngn2 has a critical and specific role in sensory neurogenesis that cannot be rescued by Ascl1⁶, but over-expression of Ngn2 in chick embryos does not up-regulate a full complement of sensory markers³²; thus more detailed analysis, for instance by transcriptome-wide comparison with neurons from a normal developing embryo³³, is required to determine the extent of sensory reprogramming.

xNgn2 and xAscl1 are the respective master transcriptional regulators of primary and autonomic neurons in *Xenopus*, and while both are able to upregulate markers from the alternative cascade, in whole embryos this upregulation is spatially confined; primary neurogenesis dorsally and autonomic neurogenesis ventrally. This is reminiscent of that reported in *Drosophila* whereby *atonal* promotes formation of external sense organs but also generates spatially restricted ectopic chordotonal organs³⁴. Again, it is currently unclear if this represents full activation of the non-endogenous cascade or perhaps a redundant function at only specific downstream targets that are more easily activated due to permissive chromatin in that anatomical region of the embryo.

In contrast to the marked lack of motor neuron gene up-regulation and the dramatic inhibition of interneuron marker in whole embryos, xNgn2 upregulates all three primary neuron subtypes in naïve ectoderm of animal caps. This indicates that patterning factors or signals in the ectoderm of the intact embryo are non-permissive for the motor neuron pathway and may suggest why reprogramming approaches for human patient-derived motor neurons from fibroblasts have required an induced pluripotent stage to erase somatic cell identity³⁵. Further exploration using the whole embryo and explant assays in *Xenopus* may help to elucidate the patterning factors and molecular determinants required for efficient motor neuron specification in the reprogramming environment.

Data availability

Raw data files available in Open Science Framework: xNgn2 induces expression of predominantly sensory neuron markers in *Xenopus* whole embryo ectoderm but induces mixed subtype expression in isolated ectoderm explants, <https://dx.doi.org/10.17605/OSF.IO/QKCFU>¹⁴.

See *Methods* section for description of data analysis and ISH scoring. Datasets presented are as follows:

- Fig1A_qPCR and Fig1B_qPCR: Mean fold change in expression for each target gene relative to uninjected controls; three independent experiments.
- Fig1C_embryos: Representative images from 49–61 embryos in each category from three independent experiments.
- Fig1D_embryos: Representative images from 36–42 embryos in each category in two independent experiments.
- Fig1E_embryos: Representative images from 31–39 embryos in each category in two independent experiments.
- Fig1F_embryos: Representative images from 21–26 embryos in each category in two independent experiments.
- Fig2A+B_qPCR: Mean fold change in expression for each target gene relative to uninjected naïve caps; four independent experiments.
- Fig2C_qPCR: Mean fold change in expression for each target gene relative to uninjected naïve caps; two independent experiments.
- Primers_qPCR: Primers used for qPCR analysis

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In their manuscript, the authors ask whether the proneural factors xNgn2 and xAscl1 induce similar neuronal subtypes when overexpressed in the *Xenopus* ectoderm. xNgn2 and downstream mediators are known in the frog to induce mainly sensory neuronal markers while xAscl1 induces predominantly autonomic neuron markers. The question asked is interesting as several studies have shown that the function of proneural factors is context dependent.

In general, the results are well presented. The data provided by the authors confirm and extend previous findings about the specific role of the two proneural factors. I thus recommend their publication in Wellcome Open Research, after some specific points and suggestions detailed below are taken into account.

In the introduction, it is stated that xNgn2 is the master regulator of primary neurogenesis and a parallel is made with what is known of its function in the development of the spinal cord and dorsal root ganglia in the mouse. It should be mentioned that Ngn1 is also expressed in these structures, in the mouse and in *Xenopus*, and that it also plays an important role in sensory neurogenesis. Relevant references should be added.

In the result section, paragraph 4, line 14 : Focusing instead of focussing.

In Figure 1A, only one specific marker of interneurons, Vsx1, is used to discriminate the identity of spinal cord interneurons despite the heterogeneity of this neuronal population. Other genes such as Evx1 and En1 could be analyzed to have a stronger overview of the role played by xNgn2 on the induction (or not) of interneuron markers.

In the mouse, the main different subtypes of sensory neurons can be discriminated based on the expression of the neurotrophic receptors ntrk1, ntrk2, ntrk3 and ret. Analyzing the ability of xNgn2 to induce their expression would strengthen the message that xNgn2 is capable of inducing sensory neuron markers and would indicate if it biases this lineage to specific subtypes or not.

In Figure 1A, Ptf1a is used as a GABAergic marker in the RT-qPCR experiments but the results obtained for this marker are not commented and it is not included in the RT-qPCR analysis in figure 2. The authors should comment the data and indicate why they found it not to be relevant for the analysis in their experiments.

In Fig. 1D,E, why not showing the same autonomic marker, Phox2a, in both Ascl1 and in Ngn2 injected embryos?

In Figure 1C-F, the percentage of embryos showing the indicated phenotype should be indicated. In embryos, xNgn2 overexpression induces mainly sensory neurons. Based on the data of Fig. 2A, the authors claims that in contrast, in AC explants, xNgn2 upregulates all three major types of primary neurons. This statement should be a little toned down as the motor neuron and interneuron markers tested in these explants are only modestly induced. Whether overexpression of Ascl1 in AC also has distinct consequences in embryos and AC could be similarly tested.

Results shown in Fig.2C could be mentioned as “data not shown”.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Developmental neurobiology

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.
